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Persistent Infection of a Lymphoma Cell Line by Herpes Simplex Virus

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The peripheral blood cells from a patient with a B-cell lymphoma were established in long-term tissue culture. Two years after establishment of the cells in culture they were infected with herpes simplex virus type 2 and the productivity and duration of viral persistence investigated. One week after infection the lymphoblastoid cells were productively infected and have remained so for a period of over 3 years. Expression of a viral glycoprotein antigen was evaluated by using a fluorescein-labeled monoclonal anti-herpes simplex virus type 2 antibody and revealed a spectrum of staining reactions grading from a lightly stippled to very intense pattern. Polymerase chain reaction analysis of the infected cells revealed the presence of the herpes simplex virus type 2 DNA polymerase gene in the infected cells that was absent from the uninfected lymphoblastoid cells. These results taken together with the long-term growth characteristics of both the infected and uninfected lymphoblastoid cells suggest that this cell line may be a good model system for studying viral infection, viral replication, viral latency, and clinical application for the isolation of human herpes virus. Am. J. Hematol. 62:93–98, 1999.

Key words: lymphoma; herpes simplex virus type 2; persistent infection; cell culture; polymerase chain reaction; karyotype

INTRODUCTION

Tissue cultured cells infected with human herpes simplex viruses (HSV) typically undergo lytic infection and do not survive in long-term culture. Henle et al. found in 15 cell lines of Burkitt's lymphoma and leukemic origin that HSV infection caused rapid (3–6 days) cell death in some instances and transient infection and survival in others [1]. A study by Rinaldo et al. demonstrated that the normal T and B lymphocytes from healthy donors supported the short-term replication of HSV type-1 (HSV-1) for 3 to 6 days [2]. Persistent HSV-1 infection of cell lines requires modification of the tissue culture conditions. Cummings et al. and Cummings and Rinaldo found that the CEM T lymphoblastoid cell line could be maintained in a chronically infected state if they helped through critical periods by lengthening the interval between subcultivation [3,4]. Modifications such as addition of anti-HSV antiserum to the culture medium [5], or phytohemagglutinin [6–8], modification of incubation temperature [9], or co-infection with a second virus [10] have been used with some success. Variations in the susceptibility of human lymphoid and myeloid cell lines to infection by HSV-1 and HSV-2 have been noted. Mizahi et al. [11], Hammer et al. [7], and Rinaldo et al. [12] recorded persistent infection by HSV-1. However, Robey et al. [13] noted susceptibility to both HSV-1 and HSV-2 in one Burkitt's lymphoma cell line, but susceptibility to only HSV-1 in another Burkitt's derived cell line.

This study was originally planned as an investigation to see if this unique cell line could support replication of cytomegalovirus and possibly serve as an in vitro model to study persistent cytomegalovirus infection. We were unable to detect a productive or a latent infection with cytomegalovirus in these cells. To see if the cell line

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would be permissive to infection with a virus of *Herpesviridae* family that is more easily cultivated in culture, HSV-2 was inoculated into the cells. HSV-2 was able to replicate in these cells. It was fortuitous that we found some older cultures still had viable cells which had previously been inoculated with the virus. This was a noteworthy result because in culture, HSV-2 is generally very cytolytic. That finding led to this investigation that demonstrates HSV-2 can be maintained in this cell line for more than 28 months.

The lymphoid cell line, which was established by placing the peripheral blood of a patient with pre-B-cell lymphoma into RPMI 1640 culture media supplemented with fetal bovine serum. The cells grow well in suspension culture and no special culture medium supplements or conditions are required. As far as we know, this cell line is the first human lymphoid cell line to show persistent in vitro HSV-2 infection extending beyond 2 years. In addition, the cells exhibit chromosomal rearrangements, possibly indicating a relationship between HSV persistence and chromosomal rearrangement. This cell line may be useful as a model for investigation of chronic, persistent HSV infection.

MATERIALS AND METHODS

Derivation of the KLC Cell Culture

A peripheral blood sample from a 27-year-old male in the leukemic phase of B-cell lymphoma was cultured in RPMI-1640 medium supplemented (GIBCO/BRL, Grand Island, NY) with 20% fetal bovine serum (Gibco BRL) in a 25 cm^2 culture flask and observed weekly for growth. The cultures were continued with change of medium or by passage once per week. At the time of collection of the sample the patient had a white cell count of 183,000/mm^2 and 67% blast cells. The blast cells in the initial sample were positive for HLA-DR, CD19, CD20, and CD21, but were negative for myeloid markers by flow cytometry, indicating that the cells are pre-B-lymphoid cells. The cells were also negative for the early antigen of Epstein-Barr virus as determined by immunohistochemical staining (anti-Epstein-Barr virus early antigen, BioGenex Laboratory, San Ramon, CA). The cell doubling time was estimated by using standard hemocytometry, and found to be between 60 to 72 hr. The cells have been growing for 3 years when this experiment was conducted, and at the time of preparation of this manuscript, the cells have been in culture for more than 5 years and are referred to as KLC cells. They also grow well in a serum-free hybridoma medium (#S-2897, Sigma Chemical Co., St. Louis, MO).

Chromosomal Analysis

Karyotypic analysis was performed by using the conventional G-banding technique on the initial blood cell sample. Once the cells were established and proliferating in culture, additional karyotypings were performed again.

HSV-2 Infection of Cells

HSV-2 isolated from a clinical sample was propagated in Hep-2 cells (American Type Culture Collection, Rockville, MD) and stored at −70°C until use. For infection the viral stock was diluted to 1×10^5 plaque forming units (PFU) per ml and a 1 ml volume was placed into a culture of the KLC cells at a cell density of 3.5×10^6 cells per ml and a viability of 95%. Production of infectious virus was documented during the first 4 days after viral inoculation and at 7, 9, 14, 18, 20, 25, and 120 days after infection of the culture by using a standard PFU assay and Hep-2 cells. During this time the cells were subcultured by transferring approximately 0.1 ml of cell culture fluid into 5 ml of the complete medium in a 25 cm^2 flask. The long-term productivity of the infected KLC cells was evaluated by using Hep-2 cell monolayers and viral plaque assay [14].

Immunohistochemical Staining of HSV-2-Infected KLC Cells

At various times after infection the KLC cells were placed onto microscope slides, air dried, fixed in acetone, and stained with a fluorescein-labeled monoclonal anti-HSV-2 antibody (#B1029-44, Baxter Diagnostics, Inc., Deerfield, IL). The stained cells were examined by using a fluorescence microscope and the percent of fluorescing cells recorded by performing counts of the cells in 10× magnified fields. Flow cytometric analysis for cell membrane markers of the infected KLC cells was compared to the uninfected cells. Both cell populations were examined for the following markers: HLA-DR, CD19, CD20, and CD21, as well as myeloid markers.

PCR Analysis for Viral DNA in Infected KLC Cells

Uninfected KLC cells and those which had been infected 3 years previously with HSV-2 were grown to a density of 3.5×10^6 cells/ml. A 1 ml volume from each cell culture was collected into a 1.5 ml microcentrifuge tube and the cell pellet collected by centrifugation at 12,000 × g for 30 sec. The cells were lysed and the cellular DNA was extracted and purified by using a DNA isolation system (Genomic DNA, Pharmacia, Inc., Piscataway, NJ). One nanogram quantities of the DNA from infected and uninfected KLC cells were mixed in separate 0.5 ml microcentrifuge tubes along with an upstream primer whose sequence is 5′-CAGTACGGCCCCGAGTTCGTGA-3′ [15] and downstream primer whose sequence is 5′-GTAGATGGTGCGGGTGATGTT-3′ as reported by Rogers et al. This primer pair amplifies a 476 bp segment of the DNA polymerase gene of HSV 1 and 2. Each amplification tube contained PCR buffer, 100

μmol/l dNTPs, Taq polymerase, and the target DNA in a volume of 100 μl. After the reactants were mixed they were heated to 99°C for 5 min to destroy any residual nuclease activity and the tubes were allowed to cool to room temperature after which 0.5 μl of Taq polymerase® (Perkin–Elmer/Applied Biosystems, Foster City, CA) was added. Amplification conditions in a thermal cycler (MJ Research, Inc., Watertown, MA) were as follows: 95°C for 5 min followed by 35 cycles of 94°C for 1.5 min, 65°C for 2 min, and 72°C for 2 min. At the end of the 35 cycles, an additional 5 min at 72°C incubation was performed as an extension step.

Amplified PCR products were analyzed on 2% agarose gels in Tris borate EDTA buffer. Twenty microliter volumes from each reaction tube were electrophoresed in gels containing ethidium bromide and examined on a gel documentation system (Eagle Eye II, Stratagene Inc., La Jolla, CA). In some experiments control HSV-2 DNA was amplified along with the unknown samples in order to insure that the PCR reaction conditions were optimal for amplification.

As shown by Rogers et al. [15], the *Ava*II restriction enzyme cleaves the 476 bp product obtained from HSV-2 with the DNA polymerase primers into two segments consisting of a 389 nucleotide fragment and an 87 nucleotide fragment. Accordingly, 10 μl volumes of the amplified template was subjected to *Ava*II digestion by placing 1 μl of the enzyme along with 2 μl of 10 × PCR buffer and 7 μl of water and incubating for 1 hr. After digestion, enzyme-treated samples and untreated samples were analyzed on 2% agarose gels and stained with ethidium bromide.

RESULTS

Morphology and Growth of the KLC Cells

It should be emphasized that we have not cloned the KLC cells in an effort to obtain a pure cell population derived from a single cell. Instead, small aliquots of the KLC cells have been passaged for a period of over 5 years at the time of the writing of this manuscript. They are routinely propagated in suspension in standard tissue culture medium on a 5 to 7 day passage schedule.

Analysis of the cellular morphology 1 1/2 years after establishment of the culture revealed a lymphoblastoid cell line of relatively homogeneous size (approximately 15 to 20 μ in diameter) having a relatively large nucleus and characteristic cytoplasmic staining by Wright's staining (Fig. 1).

Karyotypic Analysis of the KLC Cells

The peripheral blood cells which were initially isolated and analyzed for karyotype and G banding gave the following results: 48,XY,t(8;9)(q13;p24)x2, del(13)(q12q14), +18,+22. Once the KLC cells had been established in culture for 8 months, 77% of the cells displayed the following karyotypic pattern: 46,XY, t(8;9)(q13;p24)x2,del(13)(q12q14) (Fig. 2A) and 23% of the cells had the following karyotypic pattern: 46,XY, t(8;9)(q13;p24)x2,del(13)(q12q14),add(22)(q13.2) (Fig. 2B).

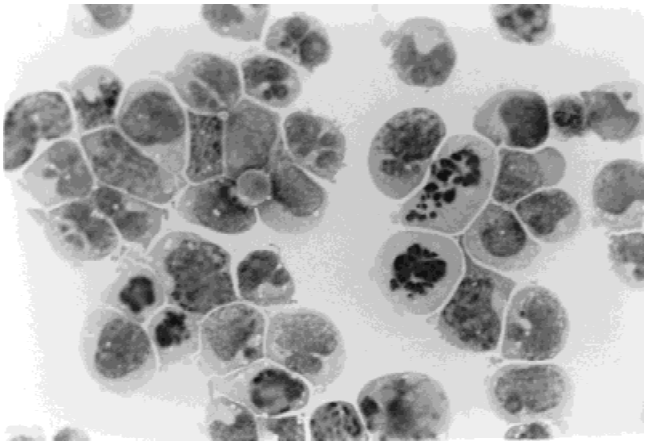

Fig. 1. A cytospin preparation of KLC cells stained with Wright's stain. The characteristic morphology and staining pattern of transformed lymphoblastoid cells is seen (1,500×).

Production of Infectious Virus by the KLC Cells

Tissue culture supernatant and cells from infected KLC cultures were collected and analyzed for infectious virus and, as well, cells were stained for the expression of viral glycoprotein antigens by an immunofluorescent staining technique.

Cytospin preparations of infected and uninfected KLC cells incubated with the fluorescein conjugated anti-HSV-2 revealed an absence of staining of the uninfected cells (not shown) and a spectrum of staining patterns in the infected KLC cells (Fig. 3). The staining pattern in the infected cells varied from cells having intensely fluorescent cytoplasm to cells displaying lightly to heavily speckled cytoplasmic staining.

Viral titer of the culture supernatant and KLC cells were determined by a plaque assay by using Hep-2 cells. After 1 week of infection the viral titer of the culture supernatant and KLC cells stabilized at 1×10^5 PFU/ml (Fig. 4). This productive HSV-2 infection in the KLC cells has continued unchanged for over 3 years at the time of preparation of this manuscript.

Determination of the percent of cells which stained positively for HSV-2 glycoprotein antigens by immunofluorescence also reveals that the percent positive cells stabilized at approximately 1 week after infection and is maintained at approximately 45% of the cells in a culture that has reached maximum cell density. This frequency of viral antigen positive cells also has been maintained

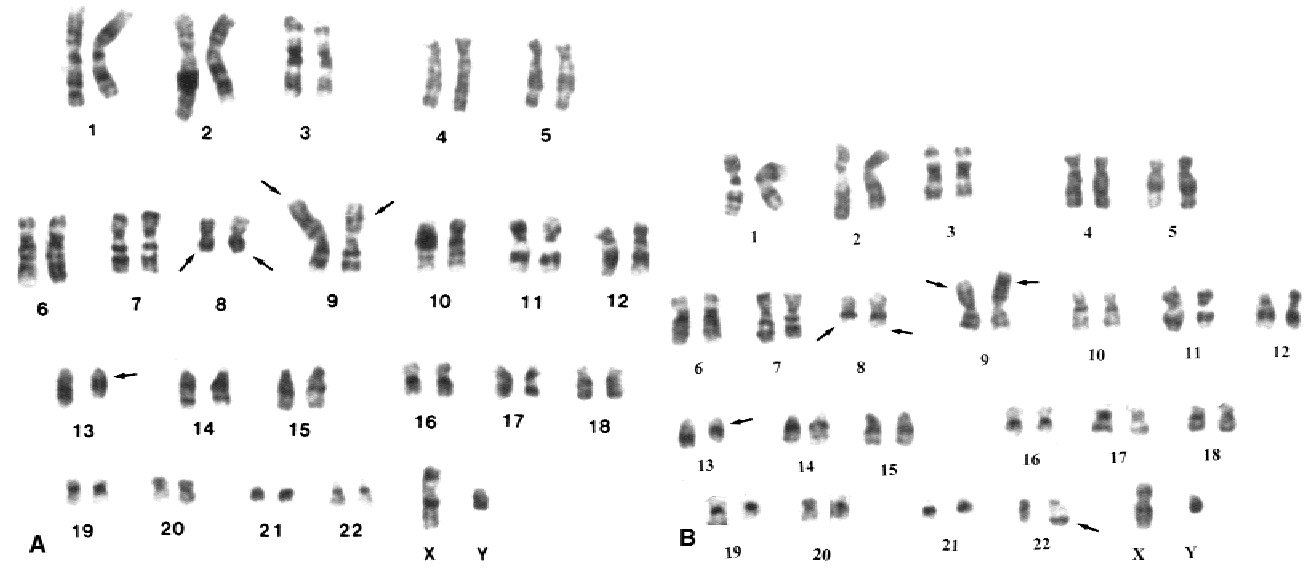

Fig. 2. Karyotype of KLC cells at the time of initiation of infection with HSV-2. Seventy-seven percent of cells displayed 46,XY,t(8;9)(q13;p24)x2,del(13)(q12q14) (A) and 23% showed 46,XY,t(8;9)(q13;p24)x2,del(13)(q12q14),add(22)(q13.2) (B).

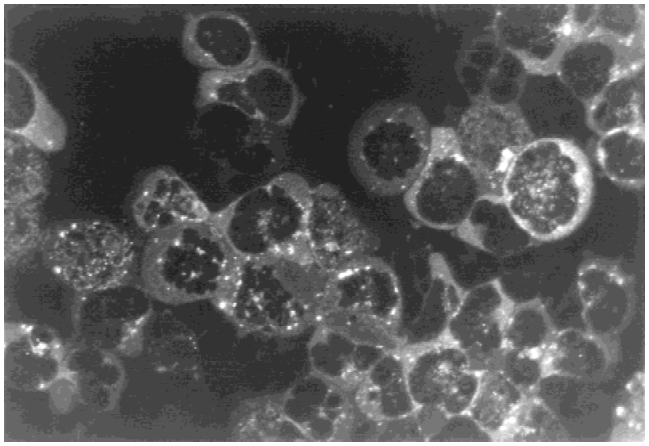

Fig. 3. A cytospin preparation of HSV-infected KLC cells stained with fluorescein isothiocyanate-conjugated mouse anti-HSV, type 2 monoclonal antibody. Some cells are brightly stained and others exhibit a stippled pattern of staining (1,500×).

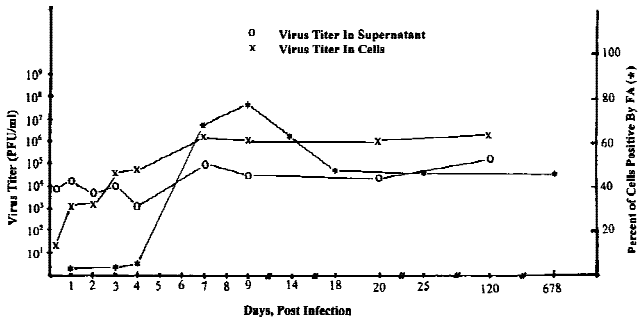

Fig. 4. Viral titers in PFU/ml of HSV-2-infected KLC culture supernatants (O) and cells (X). (*) The percent of infected cells that stained for HSV-2 glycoprotein.

for more than 3 years at the time of preparation of this manuscript (Fig. 4).

Expression of Viral DNA in Infected KLC Cells

PCR amplification of DNA from uninfected KLC cells was performed from four separate subculture generations and failed to yield a product. In contrast, the six different subculture generations of infected KLC cells all yielded the expected 476 bp product after 35 rounds of amplification (Fig. 5A). Restriction enzyme digestion of each of the 476 bp product yielded two fragments of 389 bp and 87 bp, respectively (Fig. 5B). These results were obtained in all six of the samples analyzed. *Ava*II digestion of the amplified product from a laboratory strain of HSV-2 gave identical results (Fig. 5B).

DISCUSSION

This study demonstrates the chronic, persistent infection by HSV-2 of a pre-B-lymphoma cell culture that does not necessitate unique culture conditions. The cells were not only chronically infected by the virus but also produced virus constantly for more than 25 months. These results differ from those reported in other studies in which the established cell lines were susceptible to HSV-1 but could not be readily infected and maintained as HSV-2-infected cells. Furthermore, induction of HSV infection of cells lines by various investigators was found to require the use of mitogen, interferon, or elevation of incubation temperature [5,6,8,9]. The KLC cells, re-

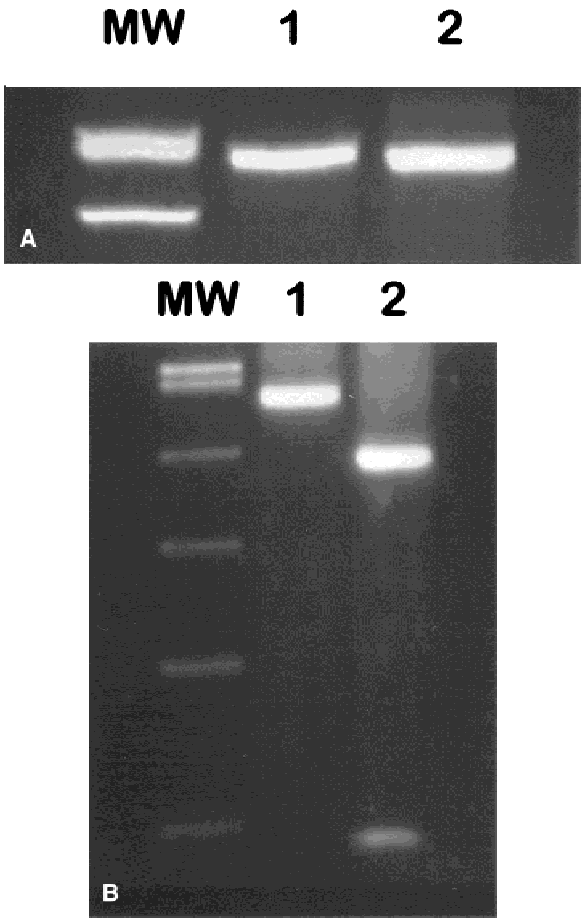

Fig. 5. PCR analysis of HSV-2 infected KLC cells. (A) MW, molecular weight markers; 525, 500, and 400. Amplification of DNA from infected KLC cells yields a 476 bp product (lane 1). HSV-2 DNA from a laboratory isolate yielded an identical size product (lane 2). (B) MW, molecular weight markers: 525, 500, 400, 300, 200, 100. Undigested, amplified HSV-2 template appears as a 476 bp product (lane 1). Restriction enzyme (*Ava*II) digestion of the amplified HSV-2 template from infected KLC cells yields two products of 87 and 389 bp (lane 2).

quired no special culture manipulation or additives in order to render the cells susceptible to induce HSV infection. Both infected and noninfected cells were cultured in the same way and both cultures showed no difference in growth behaviors (i.e., morphology, viability, and doubling time) throughout the course of the experiment. The chronicity of infection was also demonstrated by a direct fluorescent antibody technique (unpublished data). Thus, the cell line may be useful for the study of herpes viral replication and perhaps for the study of viral latency. Whether the KLC cells are susceptible to other subtypes of HSV remains to be examined. Preliminary experiments indicate that the cells are susceptible to HSV-1, but not to cytomegalovirus (data not shown). Interestingly, in the course of this experiment, the HSV-2-infected KLC cells were treated with acyclovir at 1.0 μg/ml and 10.0 μg/ml, respectively, and the viral resistance to acyclovir was observed up to 9 months of culture (data not shown). Therefore, the KLC cells will be very useful for testing new anti-viral drugs and clinically, the cells can be used for anti-viral susceptibility assay of patient isolates. These cells may also be useful for studying the effect of HSV-2 infection on subsequent infections by other herpesviruses or other viruses such as HIV, etc. Also, the KLC cells will be useful in the study of the effect of HSV infection on cytokine gene expression and on plasma membrane markers, such as major histocompatibility antigens, C3 receptors, and others.

The question remains as to why lymphocytes isolated from healthy donors can only support herpes viral infection for 3 to 6 days [2], whereas transformed lymphoid cells can support viral replication for extended periods of time [7,8,16]. It is possible that because normal lymphocytes have a relatively short life span in vitro they are not able to serve as efficient hosts for this virus compared to immortalized lymphoid cell lines. The KLC cells derived from a patient with lymphoma are negative for EBV early antigen but have distinct karyotypic abnormalities. It is possible that the karyotypic abnormalities, t(8;9) and del(13)(q12q14) not only contribute to the immortality of these cells but have may also make them permissive for chronic persistent infection by herpes viruses. In reviewing the literature of the in vitro studies with HSV by using various cell lines, the karyotypes of the cells were not given, therefore, no comparison between persistent infection and karyotype can be made. In summary, we have described a human lymphoid cell line that is permissive for chronic HSV-2 infection, and the cell line may be used in clinical laboratory for isolation of HSV-2. However, an understanding of the molecular mechanisms that render these cells susceptible to persistent HSV infection is not yet available and requires further investigation.

REFERENCES

1. Henle W, Henle G, zur Hausen H: Effect of herpes simplex virus on cultured Burkitt tumor cells and its failure to influence the Epstein-Barr virus carrier state. Cancer Res 1969;29:489–494.
2. Rinaldo CR Jr., Richter BS, Black PH, Callery R, Chess L, Hirsch MS: Replication of herpes simplex virus and cytomegalovirus in human leukocytes. J Immunol 1978;120:130–136.
3. Cummings PJ, Lakomy RJ, Rinaldo CR Jr: Characterization of herpes simplex virus persistence in a human T lymphoblastoid cell line. Infect Immunology 1981;24:817–827.
4. Cummings PJ, Rinaldo CR Jr: Coevolution of virulent virus and resistant cells as a mechanism of persistence of herpes simplex virus type 1 in a human T lymphoblastoid cell line. J Gen Virol 1989;70:97–106.
5. Hammer SM, Richter BS, Hirsch MS: Activation and suppression of herpes simplex virus in a human T lymphoid cell line. J Immunol 1981;127:144–148.
6. Nahmias AJ, Kibrick S, Rosan RC: Viral leukocyte interrelationships.

I. Multiplication of a DNA virus-herpes simplex in human leukocyte cultures. J Immunol 1964;93:69.
7. Hoggan M, Roizman B: The isolation and properties of a variant of herpes simplex produced multinucleated giant cells in monolayer cultures in the presence of antibody Am J Hygiene 1959;70:208–219.
8. Youssufian H, Hammer SM, Hirsch MS, Mulder C: Methylation of the virus genome in an in vitro model of herpes simplex virus latency. Proc Natl Acad Sci 1982;79:2207–2210.
9. Coleman V, Jawets E: A persistent herpes simplex virus infection in antibody-free cell culture. Virology 1961;13:375–377.
10. Glasgow LA Habel K: Role of polyoma virus infection and interferon in a herpes simplex virus infection in vitro. Virology 1963;19:328–339.
11. Mizrahi A, Mitchen JR, von Heyden HW, Minowada J, Moore GE: Characterization of the growth of herpes simplex virus in human lymphoid cells. Appl Microbiol 1972;23:145–154.
12. Rinaldo CR Jr, Richter BS, Black PH, Martin SH: Persistent infection of human lymphoid and myeloid cell lines with herpes simplex virus. Infect Immunology 1979;25:521–525.
13. Robey WG, Graham BJ, Harris CL, Madden MJ, Pearson GR, van de Woude GF: Persistent herpes simplex virus infections established in two Burkitt lymphoma derived cell lines. J Gen Virol 1976;32:51–62.
14. Schmidt NJ: Cell culture procedures for diagnostic virology. In: NJ Schmidt and RW Emmons, editors. Diagnostic procedures for viral rickettsial and chlamydial infections, 6th edition. Washington, DC: American Public Health Association; p 51–100.
15. Rogers BB, Josephson SL, Mak SK: Detection of herpes simplex virus using the polymerase chain reaction followed by endonuclease cleavage. Am J Pathol 1991;139:1–6.
16. Hampl W, Conrad S, Kleinschmidt AK: Herpes simplex virus type 1 long-term persistence, latency, and reactivation in infected Burkitt lymphoma cells. Arch Virol 1991;117:251–267.